BINDING OF RIBOFLAVIN AND RIBOFLAVIN PHOSPHATE BY THE PROTEINS OF MILK

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SUMMARY

The binding of riboflavin and riboflavin phosphate by the proteins of milk, particularly by α- and β-casein, has been studied. Binding constants have been determined and, from these, thermodynamic quantities have been calculated. The binding is of a low order of magnitude with an unusually high temperature coefficient. The binding by the calcium caseinate–phosphate complex in milk is equivalent to the binding by its component protein molecules. Certain similarities between the binding by the caseins, and the binding by flavoprotein enzymes, have been noted. The data indicate strongly that phenoxyl residues constitute the loci of binding.

As part of a program devoted to the study of the effect of additives on coagulation in milk it became a point of interest to know whether the calcium caseinate–phosphate complex in milk possessed solubilizing or binding characteristics peculiar to its micellar structure and distinct from the binding characteristics of its component molecules. On a priori grounds it can not be assumed that equivalence exists, for by analogy with the extraordinary solubilizing properties shown by detergent micelles, the so-called protein micelles conceivably may also have extraordinary binding characteristics. Conversely, it is conceivable, too, that the micelle may bind less substrate than its component molecules.

Riboflavin and its phosphate were selected as substrates for this study for a number of reasons. Riboflavin is a neutral molecule and its binding would not necessarily disturb the calcium caseinate–phosphate complex. A number of phenomena encountered in dairy technology are or may conceivably be related to the binding of riboflavin or riboflavin phosphates. Such for example are: (a) the photosensitizing action of riboflavin in the decomposition of ascorbic acid, and the formation of methional from methionine; (b) the partition of riboflavin between curds and whey in the manufacture of casein and cheese; and (c) the behavior of riboflavin phosphate in prolonging the time required to bring about thermal coagulation of the proteins in milk.

There are also points of general interest in studies on the binding of riboflavin and riboflavin phosphate. These pertain to the nature and number of the binding sites; to the relative binding effectiveness of various milk proteins; to the influence of the anionic phosphate group in riboflavin phosphate on binding and, finally, to the similarities which may exist between the general binding of riboflavin phosphate by proteins and the highly selective binding of riboflavin derivatives in enzyme systems.

EXPERIMENTAL PROCEDURE

Materials. α- and β-Casein were isolated from skimmilk by the method of Hipp *et al.* (5). This method depends on the difference in solubility in aqueous urea. α-Casein was found to be electrophoretically pure at pH 8.6. β-Casein contained 5% α-casein as an impurity. Electrophoretically pure β-casein was not realized with the urea method. The removal of the 5% α-casein impurity, however, was effected by the method of Warner (15), based on the different solubilities of these proteins in aqueous solutions at low temperatures. α-Casein prepared by the urea method dissolved in NaOH to yield turbid solutions. The particles responsible for the turbidity were removed. The α-casein preparation dissolved by means of borax in a 50% alcoholic solution, 0.15 *M* in NaCl, was fractionally precipitated with alcohol. The large particles precipitated first and, following their removal by filtration, the main body of α-casein was precipitated from the filtrate by the addition of water and acid. β-Lactoglobulin prepared according to Palmer [see Gordon *et al.* (4)] was recrystallized four times. Bovine serum albumin was a commercial product which had been recrystallized twice.

Riboflavin of U.S.P. quality was recrystallized in the manner prescribed by Pasternack and Brown (11). Riboflavin -5′-monosodium phosphate dihydrate, a Hoffmann-LaRoche product,[1] in paper electrophoresis exhibited two faint bands in addition to the major band belonging to the phosphate.

Methods. Equilibrium dialysis furnished data for the calculation of moles solute bound per 10^5 g. protein (12). The 7.5-ml. protein solution within the cellophane bag contained 0.188 g. protein. The bag was submerged in 7.5 ml. of either a buffer or salt solution contained in a screw-capped vial. A glass marble within the bag facilitated mixing during the slow end-over-end motion of the vial during a three-day equilibration period, usually at 4°. The bags, following loading with sample, were carefully tied so that the membranes were approximately under the same tension, and the presence of air bubbles was minimized. This done, the weighing of the bag before and after dialysis permitted conclusions to be drawn in certain instances concerning the manner in which the number-average particle weight of the protein was affected by changes in temperature and solution composition. Binding data pertaining to riboflavin phosphate required the application of a correction factor to compensate for the uneven distribution of ions brought about by the Donnan effect.

The fully developed equation based on Donnan ratios for the calculation of the correction factor requires knowledge of the volumes of the equilibrated solutions inside and outside the bag. However, approximation equations in which these volumes do not appear explicitly may be used to advantage (12).

Basically, the full equation permitted the calculation of the concentration of positive ions inside and outside the bag, thus:

[1] It is not implied that the U. S Department of Agriculture recommends the above company or its product to the possible exclusion of others in the same business.

$$m' = \sum \frac{(M-m)^{\nu_r}\ \nu_r\ M_r}{(M-m')^{\nu_r} + (P+m')^{\nu_r}\left(\frac{V_a}{V_i}\right)^{\nu_r+1}}$$

M—total moles of cations associated with buffer salts and protein
m′—total moles of cation within the bag
P—equivalents of protein within the bag
ν_r—valence of anion of species r
V_a, V_i—volumes of solution outside and inside bag, respectively
M_r—total moles of anion of species r

The summation is taken over all anionic species present in the system. The equation may be solved graphically for m′, and the cationic concentration inside and outside the bag may then be calculated. This done, the equilibrium riboflavin phosphate concentration may be calculated from the Donnan ratios (2).

Dialysis experiments conducted at 20° C. required the employment of aseptic techniques. Protein and riboflavin solutions were sterilized by filtration; and membranes, thread, pipettes, and implements were sterilized by steam.

Concentrations of riboflavin and riboflavin phosphate were determined by absorbance measurements at 445 mμ. The magnitude of the error at the lowest concentration was of the order 0.5%; the difference in concentration was about 30%.

In experiments to determine the binding by the protein complex of milk, milk was dialyzed against its dialyzable constituents (prepared by dialyzing water against a large excess of milk), supplemented either with riboflavin or riboflavin phosphate. Casein was determined according to the method recommended by the A.O.A.C. (10).

Phosphate buffer solutions were prepared from K_2HPO_4 of analytical grade. pH determinations were made by means of a glass electrode. In studies pertaining to the effects of copper, zinc, iron, and molybdenum, the outside solutions were adjusted to contain one of the following salts: $CuSO_4$, $ZnSO_4$, $FeCl_3$, and $(NH_4)_2MoO_3$ in 0.001 *M* concentration. In studies on the effect of Ca^{++}, the protein solutions were adjusted to contain 0.005 mole $CaCl_2$ per liter. All solutions were made 0.4 *M* with respect to $NaNO_3$. To study the effect of urea, both the protein and outside solutions were adjusted to contain 6.6 *M* urea and 0.1 *M* phosphate buffer of pH 6.75.

The binding isotherm for riboflavin. Table 1 shows how the binding of riboflavin by α- and β-casein in phosphate buffer of pH 6.75 and ionic strength 0.2 varied with equilibrium concentration. A straight line passing through the origin defined the relationship with sufficient exactness in the concentration range specified in the table. The constant of proportionality may be designated either as an apparent association constant or as a partition coefficient.

The magnitude of the binding is quite small. The binding by α- and β-casein, respectively, at an ionic strength of 0.2, was characterized by association constants, the values of which were 1.7 and 1.3×10^3 liters per 10^5 g.

Influence of temperature on the association constant. Table 2 shows how the binding of riboflavin decreased two- to threefold as the temperature was

TABLE 1

Binding of riboflavin by α- and β-casein in phosphate buffer

Protein	Equil. concn. riboflavin [R] moles per liter × 10^5	Riboflavin bound, r moles per 10^5 g.	$\frac{r}{[R]}$ liters per 10^5 g.
α-casein	1.7	0.029	1,700
	2.3	0.039	1,700
	3.5	0.060	1,720
	6.2	0.110	1,770
	6.2	0.105	1,690
	8.7	0.152	1,740
β-casein	2.0	0.026	1,300
	2.7	0.034	1,260
	3.9	0.050	1,290
	6.7	0.086	1,280
	9.4	0.122	1,300

pH—6.75.
Ionic strength—0.2.

raised from 4 to 55° C. The association constant belonging to β-casein, for example, decreased from 1.3×10^3 liters per 10^5 g. at 4° to 4.7×10^2 liters per 10^5 g. at 55° C. Knowledge of the values of the association constant at different temperatures permitted the calculation of the change in enthalpy accompanying the binding. Change in enthalpy, $\triangle H$, was not constant over the temperature range 4 to 55°, but varied in some manner with temperature. In the absence of sufficient data to define this relationship, it has been assumed that $\triangle H$ is independent of temperature in the temperature range 4 to 20°, and accordingly the values of $\triangle H$ belonging to α- and β-casein in this temperature range have been calculated. Much the same assumption is made in the calculation of the values of $\triangle H$ which appear in the literature and, hence, a basis for comparison exists. $\triangle H$ values of −5.8 and −4.8 kcal. per mole were obtained, respectively, for the binding by α- and β-casein. From the value of the association constant for β-casein, with a molecular weight of 25,000, the change in free energy at 4° of −3.1 kcal. per mole, and the change in entropy of 6 cal. per mole per degree, was calculated. As the temperature was raised from 20 to 55°, the decrease in the values of k was less than one would anticipate

TABLE 2

Variation in the value of the association constant belonging to the casein-riboflavin complex with variations in temperature

Protein	α-Casein			β-Casein		
Temp. ° C.	Association const. K *(liters per 10^5 g.)*	Av. enthalpy change (−△H) *(cal. per mole)*	Osmosis [a] index *(g.)*	Association const. K *(liters per 10^5 g.)*	Av. enthalpy change (−△H) *(cal. per mole)*	Osmosis index *(g.)*
4	1,700		+0.4	1,300		+1.1
		5,800			4,800	
20	950			800		
55	820		+1.1	570		+0.4

[a] Osmosis index—algebraic difference of increase in weight of bag containing no protein, and of bag containing protein.

had $\triangle H$ remained constant. The indices given in the last column relating to the quantity of water diffusing through the dialyzer membrane as the result of osmosis showed that the number of α-casein particles increased and the number of β-casein particles decreased as the temperature was raised from 4 to 55°. This change in the state of aggregation with changes in temperature could conceivably influence the binding in the direction indicated by the data.

The influence of ionic strength, pH, metals, and urea on the association constant. The association constant for the binding of riboflavin by both α- and β-casein in phosphate buffer, pH 6.75, was not influenced by changes in ionic strength from 0.1 to 0.5. The values of the association constant changed slightly with a change in pH from 6.2 to 7.7. No mediating effect was observed for cations such as Cu^{++}, Fe^{+++}, and Zn^{++}, which form chelates with riboflavin and complexes with α- and β-casein. A slight competitive effect of Cu^{++} and Zn^{++} was observed in β-casein solutions. No mediating effect was observed for either calcium or molybdenum.

Binding of riboflavin was seriously weakened in the presence of urea. The association constant for the binding by α-casein in 6.6 *M* urea solution was 180 liters per 10^5 g.—a value approximately 10% of that observed in the absence of urea. Similarly, the value of association constant for the binding of β-casein in 6.6 *M* urea was approximately 6% of that observed in the absence of urea.

The indices relating to osmosis showed a strong disaggregation effect of urea on α- but not on β-casein. This is consistent with observations reported in the literature to the effect that β-casein is monodisperse in neutral aqueous solutions and that α-casein is polydisperse (15).

Binding of riboflavin by the calcium caseinate—calcium phosphate complex in milk. In Figure 1, two curves are compared—one representing the binding of riboflavin by a mixture containing four parts of sodium α-caseinate and one part sodium β-caseinate, and the other representing the binding by the calcium caseinate–calcium phosphate complex in milk. The quantity of bound riboflavin was approximately the same in the two systems, showing that neither the micellar structure nor bound calcium mediated the binding. The failure of calcium to mediate the binding has already been indicated. The complex in milk is said to be associated with colloidal $Ca_3(PO_4)_2$; it may easily be shown experimentally that $Ca_3(PO_4)_2$ does not bind riboflavin.

The binding of riboflavin phosphate by α- and β-casein. The data pertaining to the binding of riboflavin phosphate contain an element of error, in that a large correction has been applied to compensate for the uneven distribution of anions brought about by the Donnan effect. In view of this, the data are significant only with respect to the order of magnitude of the values involved. The much greater solubility of riboflavin phosphate compared with riboflavin permitted the extension of the binding data to much greater equilibrium concentrations and, hence, a more complete characterization of the binding curve was realized.

In Figure 2, points representing the ratios between riboflavin phosphate bound per 10^5 g. and the equilibrium concentration were plotted as ordinates,

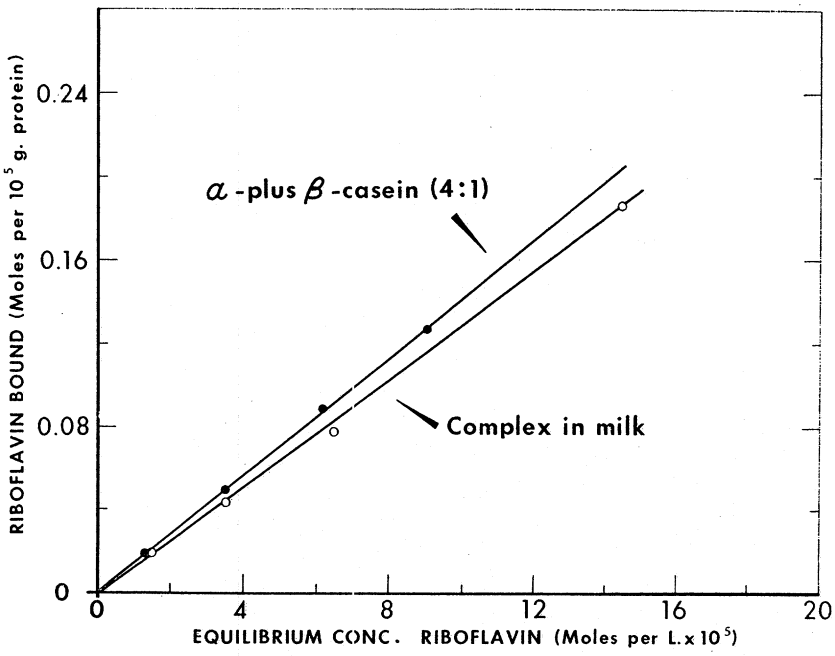

FIG. 1. The binding of riboflavin by the calcium caseinate–calcium phosphate complex in milk, and by a solution containing sodium α- and β-caseinates in the ratio 4: 1.

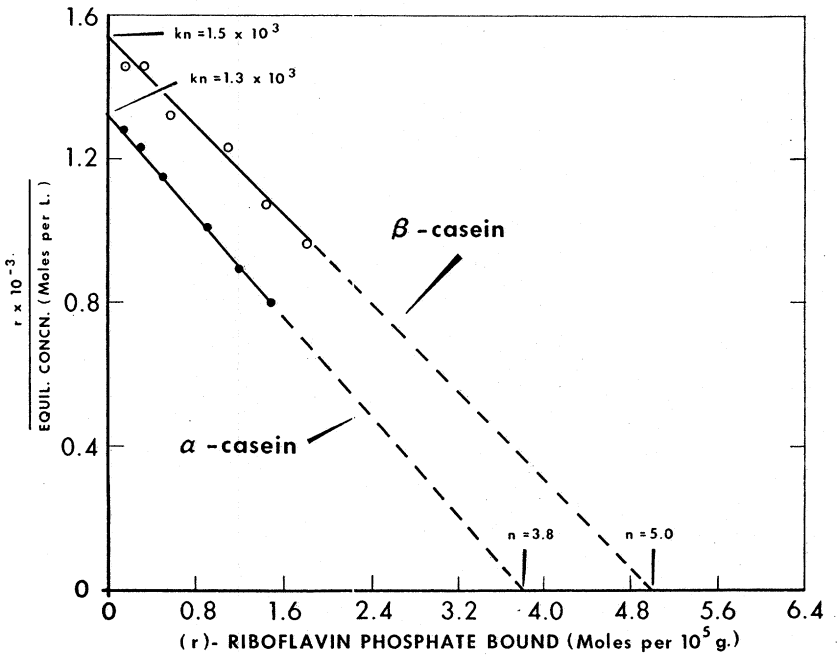

FIG. 2. The binding of riboflavin phosphate by α- and β-casein in phosphate buffer pH 6.75, ionic strength 0.2.

and points representing moles bound were plotted as abscissas. Reasonably good lines permitting extrapolation were drawn through the points. The lines belonging to β-casein and α-casein, respectively, intersected the abscissa axis at points representing a maximum of five moles riboflavin bound per 10^5 g. β-casein and about four moles bound per 10^5 g. α-casein.

The lines belonging to α- and β-casein intersected the ordinate axis at points representing the respective apparent association constants, 1,300 and 1,500 liters per 10^5 g.

The effect of temperature on the binding of riboflavin phosphate. Table 3 shows how the apparent association constant for the binding of riboflavin phosphate varied with temperature. The rate at which the association constant

TABLE 3

Influence of temperature on the binding of riboflavin prosphate by α- and β-casein in phosphate buffer, pH 6.75, ionic strength 0.2

Protein	α-Casein		β-Casein	
Temp. °C.	Association const. *(liters per 10^5 g.)*	Enthalpy change ($-\Delta$H) *(cal. per mole)*	Association const. *(liters per 10^5 g.)*	Enthalpy change ($-\Delta$H) *(cal. per mole)*
4	1,300	4,900	1,500	7,700
20	800		700	
55	450		350	

changed with temperature had the same magnitude as the rate of change observed for the binding of riboflavin.

The binding of riboflavin and riboflavin phosphate by various proteins. In Table 4, the association constants are given for the binding of riboflavin phosphate by α- and β-casein, native and denatured bovine serum albumin, and native and denatured β-lactoglobulin. The following order with respect to binding affinities was observed: α-casein, β-casein or bovine serum albumin, β-lactoglobulin.

Denaturation effected a significant increase in the binding capacity of β-lactoglobulin. Thus, a certain openness in structure was required for maximum binding efficiency.

TABLE 4

Binding of riboflavin and riboflavin phosphate by various proteins at pH 6.75, ionic strength 0.2

Protein	Association const. (riboflavin) *(liters per 10^5 g.)*	Association const. (riboflavin phosphate) *(liters per 10^5 g.)*
α-casein	1,700	1,300
β-casein	1,300	1,500
Bovine serum albumin	1,000	1,100
Bovine serum albumin (denatured)	1,200	1,200
β-lactoglobulin	300	100
β-lactoglobulin (denatured)	1,100	300

The influence of metals and ionic strength on the binding of riboflavin phosphate. As in the binding of riboflavin, Ca^{++}, Cu^{++}, Zn^{++}, Fe^{+++}, and Mo^{++} failed to mediate the binding. Pertinent data are omitted because of their negative character.

In the range of ionic strength 0.5 to 1.0, a range in which the Donnan effect is largely suppressed, no significant competitive effect on the part of buffer anions was observed. α-Casein but not β-casein was effectively salted out of phosphate buffer solutions at pH 6.75 and ionic strength 1.0.

The binding of riboflavin phosphate by the calcium caseinate–calcium phosphate complex in milk. Binding data, obtained in the equilibrium dialysis of skimmilk against its diffusable constituents augmented with riboflavin phosphate, would appear superficially to indicate that the percentage of riboflavin phosphate bound by the complex is greater than the percentage bound by an equivalent weight of the sodium caseinates at pH 6.75. However, the Donnan effect is reduced in milk because of the binding of Ca^{++}. Application of the Donnan correction showed that the apparent association constants were the same: 1,400 liters per 10^5 g. In calculating the Donnan correction the ionic strength of milk was taken as 0.075 (17), and it was assumed that 1.04% calcium was bound by casein (1).

DISCUSSION

Knowledge of the composition of the calcium caseinate–phosphate complex in milk and the binding constants of its component proteins permits the calculation of the quantity of riboflavin or riboflavin phosphate bound by the complex. The complex in milk is a loose, easily penetrated aggregate with no characteristic binding attributes such as a micellar or globular structure conceivably might possess. Electron microscopy discloses the complex as individual, spherical particles. Because the resolving power of the electron microscope is not great enough, fine structure is not revealed. The data on binding, serving to supplement the information obtained with the electron microscope, show that the micelle in milk is sufficiently porous to invite into its interior large neutral organic compounds.

The complex, although globular in appearance, behaves as a moiety of extended particles. Fine structure rather than shape is the feature which distinguishes one form of a binding entity from another. Thus, β-lactoglobulin in its globular undenatured form binds only insignificant amounts of riboflavin; the extended denatured form, however, binds to the same degree as bovine serum albumin and β-casein.

Because of its low solubility, riboflavin is available for binding studies only in a limited concentration range. A unique interpretation of binding data is, therefore, not possible. The data lend themselves to interpretation with a minimum degree of bias, either in terms of the participation of a small number of identical binding sites with small association constants, or in terms of solution theory as a distribution of riboflavin between two phases—an aqueous and a protein phase. The interpretation in terms of identical binding sites will be considered in another portion of this discussion. Salting out of α-casein is not

attended by any significant salting-out or salting-in of riboflavin or its phosphate. The binding of these solutes has, therefore, a phase distribution character; that is, each protein molecule as it binds riboflavin and riboflavin phosphate behaves as if it were a part of a separate phase.

Certain aspects of anion binding are not observed in the binding of riboflavin and its phosphate. The association constants for the binding of riboflavin are of the same order of magnitude as that observed by Klotz and Walker (9) in the binding of sulfanilamide by bovine serum albumin. The pKa values are approximately the same, justifying the conclusion superficially that it is the anion of riboflavin which is bound. This conforms with the conclusions of Klotz and Walker concerning the influence of pKa on the binding of sulfonamides. However, there are significant points of difference between the binding of riboflavin and the binding of anions. The order with respect to the affinity of the various proteins for riboflavin is quite different from the order (8) with respect to their affinity for anions (see Table 4). The large enthalpy change (Table 2 and 3) connected with the binding of riboflavin and its phosphate is atypical of anion binding (6). Failure of the doubly charged phosphate group in riboflavin phosphate to contribute substantially to the degree of binding (Table 3) and absence of competition between buffer anions and riboflavin phosphate are also atypical of anion binding.

Although the order with respect to the affinity of native milk proteins for riboflavin is essentially the same as the order with respect to the binding of Cu^{++} (3), no evidence was obtained of trace metal mediation in the binding of riboflavin and its phosphate.

Urea in large concentrations displaced riboflavin from both α- and β-caseins, but displacement surprisingly was not complete even in 6.6 *M* urea solutions. Klotz, Triwush, and Walker (7) have shown that the displacement of the strongly bound anionic dye methyl orange from serum albumin is measurably complete in 6.6 *M* urea.

The binding data lend themselves to a reasonable interpretation based on the presence in each of the various proteins of a single set of identical binding sites. The selection of these binding sites is based on the hypothesis that the ureide-carbonyl, and the aromatic-amine groupings of the riboflavin molecule, will seek out preferentially the most complementary groups on the protein molecule. This condition is met if the phenoxyl, that is, the tyrosine residues, are the loci of binding. This residue contains a hydrogen-donor hydroxyl group attractive to the ureide-carbonyl group of riboflavin, and the residue as a whole is attractive to the aromatic-amine grouping.

The respective number of phenoxyl groups (see Table 5) present in 10^5 g. of α-casein, β-casein, bovine serum albumin, and β-lactoglobulin (14) is 45, 18, 28, and 20, respectively. The order with respect to affinities (Table 4) is, therefore, not the same as the order with respect to the number of phenoxyl groups. Additional assumptions are required. It is doubtful that all of the phenoxyl groups are free to bind riboflavin; interaction between the hydroxyl and the combined carboxyl and cationic groups on the protein molecule may

TABLE 5

Calculated ordar of relative affinity of various proteins for riboflavin based on free phenoxyl groups

Protein	Cations	Anions	Phenoxyl	Phenoxyl (free)
α-casein	104	102	45	11.0
β-casein	84	81	18	5.0
Bovine serum albumin	148	139	28	5.0
β-lactoglobulin	104	142	20	4.0

take place. Klotz and Urquhart (8), in their attempt to establish a configurational basis for anion binding affinities, considered this internal interaction. An approximation to the number of free phenoxyl groups can be made on the basis of the following reasonable assumptions:

(a) hydrogen is bonded with the same intensity to anionic and cationic groups (small differences would complicate the mathematical treatment without altering conclusions);
(b) internal binding is governed by the laws of mass action; and
(c) there is an average of five phenoxyl groups per 10^5 g. of β-casein which are free to bind riboflavin (see Figure 2).

Based on these assumptions, the following relationships may be derived, thus:

$$N_{free} = \frac{N_{total}}{k \triangle N + 1}$$

$$k = \frac{N_{total} - 5}{5 \triangle N} = 0.016$$

N_{free} is the number of free phenoxyl residues, N_{total} the total number, $\triangle N$ the difference between the sum of the number of cationic and anionic groups and the number of phenoxyl groups, and k is an affinity constant common to all proteins. The values in the last column of Table 5 show the calculated number of phenoxyl residues free to bind riboflavin, and it is apparent that the order with respect to these binding sites is essentially the same as the order of binding affinities observed experimentally. Not only is there qualitative agreement but the relative indices agree quite well with the relative maximum values of the association constants. With employment of strong urea solutions as solvent the ratio between the association constants belonging to α- and β-casein approaches quite closely the ratio of 2.2:1 between the free phenoxyl groups. This observation may mean that the binding sites in the polymeric form of α-casein are not as available as they are in the monomeric form.

Some comment is apropos concerning the failure of the phosphate grouping to contribute significantly to the binding. Theorell and Nygaard (12) have shown that the association constants for the binding of riboflavin and riboflavin phosphate, respectively, by Warburg's yellow apoenzyme are 10^2 liters per mole and 10^8 liters per mole. Thus, the order of magnitude of the constant for the binding of riboflavin is the same as that observed in the binding of riboflavin by milk proteins. The order of magnitude, however, for the binding of riboflavin phosphate is about 10^6 times greater. Theorell and Nygaard have postulated, in view of their observation, that only the doubly charged phosphoric

acid residue is reactive, that firm binding of riboflavin phosphate is possible only under conditions in which two positively charged groups on the protein molecule are in close proximity. This reasoning applied to the data on milk proteins leads to the conclusion that, in these proteins, two positively charged groups would not be found in juxtaposition with each other and with a tyrosine residue.

The large value of $-\triangle H$, 11,700 cal. per mole, observed in the enzymic binding of riboflavin phosphate, may be presumed to represent a nonionic contribution to binding inasmuch as in typical anion binding $-\triangle H$ is very small (6). Hence, it is a matter of interest to compare the thermodynamic data pertaining to the riboflavin–β-casein system with data on the riboflavin phosphate–apoenzyme complex. The value of $-\triangle H$, 4,800 cal. per mole, compared with the value 11,700 cal. per mole associated with the binding in Warburg's yellow enzyme, suggests that in the enzyme at least two tyrosine residues are cooperatively binding riboflavin. The change in entropy, which is positive in both the riboflavin, β-casein, and the riboflavin phosphate, apoenzyme systems, about 6 and 4 cal. per mole per degree, respectively, also points to a resemblance in the character of the interactions.

Although the magnitude of the binding by milk proteins is quite small, the quantity of riboflavin bound at low temperatures in milk comprises 25-30% of the total riboflavin. The partition of riboflavin between curds and whey in the manufacture of various products would be determined by the relationships presented in this paper. The employment of relatively high temperatures would favor the retention of riboflavin in milk serum. In a subsequent paper it will be shown that the photochemical decomposition of riboflavin is inhibited to some extent by casein, and that the degree of inhibition may be related to the degree of binding.

Attractive as the mechanism based on the participation of a small number of identical binding sites appears to be, the data presented in this paper do not effectively rule out other mechanisms. Viewing, for example, the binding of riboflavin phosphate as a partition of the phosphate between two phases, one may postulate that the decrease in the value of the partition coefficient at the higher concentration levels is an indication of changes in the activity coefficient of the phosphate brought about by dimerization.

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